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13. ABSTRACT *(Maximum 200 Words)*

Complement is a major effector mechanism of the immune system. Membrane complement inhibitors on the surface of breast tumor cells, may play a crucial role in determining tumorigenesis and the outcome of antibody-mediated immunotherapy. The objective of this proposal is to determine the role of complement and complement inhibitors in tumorigenesis and to determine whether reversing the effects of tumor-expressed complement inhibitors will allow effective immune-mediated clearance of tumor cells.

We have established rodent models of human cancer that are suitable for evaluating the role of complement and complement inhibitors in the growth and control of breast cancer (and is likely applicable to many types of cancer). We have shown for the first time in vivo that a complement inhibitor expressed on a tumor cell surface can promote tumor growth, and have shown that different complement-associated effector mechanisms can operate to control tumor growth.

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INTRODUCTION

Complement is one of the major effector mechanisms of the immune system and membrane complement inhibitors on the surface of breast tumor cells, may play a crucial role in determining tumorigenesis and the outcome of mAb-mediated immunotherapy. The objective of this proposal is to determine the role of complement and complement inhibitors in tumorigenesis and to determine whether reversing the effects of tumor-expressed complement inhibitors will allow effective immune-mediated clearance of tumor cells. Complement inhibitors are species selective, and human complement inhibitors are not effective against rodent complement. The work proposed is based on developing rodent models of human breast cancer in which human tumor cells are transfected with rodent complement inhibitors. The models will be used to determine whether complement inhibitors expressed on the surface of breast tumor cells modulate tumor progression *in vivo*, both in the presence and absence of breast tumor-specific complement-activating antibodies, and whether blocking the function of tumor-expressed complement inhibitors will effect the growth of tumor cells *in vivo*.
of human breast cancer.

Please note that this report covers only a 6 month period. The PI changed institutions and there was a hiatus in funding and the re-establishment of research program.

BODY

Training: During the period of the award the PI has been trained in and has gained hands on experience in rodent models of breast cancer. The award has also provided for the PI to spend the time to become better trained in tumor immunology (theory and practice).

TASK 1: Months 0-6: Preparation of DNA constructs and vectors for expressing mouse and rat complement inhibitors Crry and CD59.

This task has been completed. Constructs containing rat CD59 and Crry were prepared in the mammalian expression vector pCDNA3.

TASK 2: Months 6-24: Transfection of breast cancer cell lines with cDNA encoding rodent complement inhibitors (Crry, and CD59), selection of high expressing clones, and determination of the susceptibility of transfected and untransfected cell lines to human and rodent complement *in vitro*.

This task has been largely completed and extended. In addition to the breast tumor cell lines MCF7 and BT474 (in proposal), an additional breast tumor cell line, SKBR3, and a neuroblastoma cell line, LAN-1, have been transfected. The neuroblastoma cell line was included to determine if complement-effector mechanisms against breast cancer cells is similar or different for other types of tumor, and may lead to a better understanding of complement mechanisms involved against breast tumor cells.

The characterization of the BT474 and SKBR3 cells lines was presented in the previous progress report. Preliminary data with transfected LAN-1 and MCF7 cells lines was also previously presented. Detailed characterization of MCF7 and LAN-1 cells has now been peformed and the data has been published [2] or submitted [3] (paper and manuscript included).

TASK 3: Months 18-36: Determine the *in vivo* tumor growth rate in nude rats of untransfected breast cancer cells, and cells expressing various rodent complement inhibitors (estimate 100 rats required).

This task has been completed and the data published [2] or submitted [3].

TASK 4: Months 18-36: *In vitro* analysis of control and transfected tumor cells isolated from tumors grown in rats. Analysis of continued complement inhibitor expression, complement deposition, and *in vitro* susceptibility to complement.

Task partly completed and data published [2] or submitted [3]. We have also demonstrated that growth of MUC1 positive MCF7 cells in nude rats elicits an IgM response. This has some similarities to an IgM response to MUC1 often observed in humans. This raises the possibility that this normally ineffective response in humans may be made more effective with the blockade of complement inhibition at the tumor cell surface. In vitro susceptibility of tumor-derived cells has not yet been performed.

TASK 5: Months 18-36: Determine the effect of blocking mouse Crry and CD59, expressed on human breast tumor cells, on the susceptibility of transfected human tumor cells to rat complement *in vitro*.

This task has not yet been initiated. We are in the process of transfecting tumor cells with mouse complement inhibitors in order to begin in vivo experiments (in experiments above, we used human cells transfected with rat complement inhibitors).

TASK 6: Months 24-48: Determine the effect of administering complement activating, tumor specific anti-HER2 and/or anti-MUC1 antibodies to rats bearing control and transfected tumors (estimate 60 rats required). Analysis of isolated tumor cells.

Not yet initiated

TASK 7: Months 30-48: Determine the effect of blocking mouse Crry and CD59, expressed on human breast tumor cells, on the growth of transfected human tumor cells in nude rats (estimate 100 rats required). Analysis of isolated tumor cells.

Not yet initiated

KEY RESEARCH ACCOMPLISHMENTS

- Shown that the expression of either rat CD59 and rat Crry on human breast cancer cells and a neuroblastoma cell line protects them from lysis by rat complement. Used transfected human tumor cell lines to show directly that complement inhibitors expressed on tumor cells provide effective protection from complement-mediated lysis.

- Confirmed the species selectivity of human complement inhibitors expressed on breast and neuroblastoma tumor cells.

- Established rat model of human cancer that is relevant for the study of complement. Demonstrated for the first time in vivo that a complement inhibitor expressed on a tumor cell surface can promote tumor growth. Demonstrated that different complement-associated effector mechanisms are operative in different models of cancer; direst C5b-9-mediated lysis of tumor cells in neuroblastoma (LAN-1) model, and C3 opsonization and cell-mediated cytolysis in breast cancer (MCF7) model.

REPORTABLE OUTCOMES

Publications, abstracts and presentations:

- 1. Caragine, T., Chen, S., Frey, A.F., Cheung, N.K. and Tomlinson, S. (2000). Expression of a complement inhibitor on the surface of MCF7 breast cancer cells promotes tumor growth. Era of Hope: Department of Defense Breast Cancer Research Program, Atlanta, June 8th-12th, 2000.

- 2. Chen, S, Caragine, T., Cheung, N, K. V. and Tomlinson, S. (2000) CD59 Expressed on a Tumor Cell Surface Modulates DAF Expression and Enhances Tumor Growth in a Rat Model of Human Neuroblastoma. *Cancer Res.*, **60**, 3013-3018.

  3. Caragine, T., Okada, N., Frey, AF and Tomlinson, S. (2001). A tumor expressed inhibitor of the early but not late complement pathway suppresses an anti-tumor response in a rat model of human breast cancer. *Submitted (Cancer Research)*

Cell Lines

- Breast cancer cell lines (MCF7, BT474 and SKBR3) and a neuroblastoma cell line (LAN-1) stably expressing rat CD59 and rat Crry complement inhibitors have been developed.

CONCLUSIONS

We have established rodent models of human cancer that are suitable for evaluating the role of complement and complement inhibitors in the growth and control of breast cancer (and is likely applicable to many types of cancer). We have shown for the first time in vivo that a complement inhibitor expressed on a tumor cell surface can promote tumor growth, and have shown that different complement-associated effector mechanisms can operate to control tumor growth.

Our data demonstrating that an anti-MUC1 IgM response occurs in nude rats and may play a role in activating complement and controlling tumor growth [3] raises the possibility that neutralization of complement regulatory proteins may enhance a normally ineffective cytolytic humoral immune response observed in some (eg MUC1 positive) cancers, even without the exogenous administration of anti-tumor antibodies.

[CANCER RESEARCH 60, 3013–3018, June 1, 2000]

CD59 Expressed on a Tumor Cell Surface Modulates Decay-accelerating Factor Expression and Enhances Tumor Growth in a Rat Model of Human Neuroblastoma[1]

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ABSTRACT

It has been hypothesized that complement inhibitors expressed on the surface of tumor cells prevent effective immune-mediated clearance. Whereas there are *in vitro* data to support this hypothesis, the species-selective activity of complement inhibitors has been a hindrance to investigating the role of membrane-bound complement inhibitors in rodent models of human cancer. The CD59-positive LAN-1 human neuroblastoma cell line was significantly more sensitive to lysis by rat complement than by human complement, illustrating the species selectivity of endogenously expressed complement inhibitors. Transfection of LAN-1 cells with rat CD59, an inhibitor of the terminal cytolytic membrane attack complex, effectively protected the cells from lysis by rat complement *in vitro*. When LAN-1 cells stably expressing rat CD59 were inoculated into immune-deficient rats, the onset of tumor growth and the rate of tumor growth were significantly enhanced compared with those of control-transfected LAN-1 cells. These data show directly that the expression of a complement inhibitor on a tumor cell promotes tumor growth. Flow cytometric analysis revealed that the endogenous expression of decay-accelerating factor (DAF), an inhibitor of complement activation, was up-regulated on the surface of cells after *in vivo* growth. Of further interest, higher levels of DAF were present on CD59-transfected cells than on control-transfected cells derived from tumors. Increased DAF expression correlated with decreased complement deposition on the tumor cell surface. These results show that expression of complement inhibitors on a tumor cell has functional consequences with regard to complement deposition *in vivo* and indicate that CD59 can indirectly effect complement activation and C3 deposition *in vivo* via a link between CD59 and DAF expression.

INTRODUCTION

Normal cells are protected from inappropriate complement attack by membrane-bound complement-inhibitory proteins that either prevent complement activation or block the formation of the terminal cytolytic MAC.[3] Tumor cells also express complement-inhibitory proteins, sometimes at elevated levels, and provide tumor cells with protection from complement-mediated injury. Blocking the function of complement inhibitors expressed on the surface of tumor cells may allow effective immune-mediated clearance of some tumors and improve prospects for immunotherapy using complement-activating antitumor antibodies. Complement effector mechanisms that may be involved in host response to tumor cells include the activation and amplification of an inflammatory response, recruitment of immune effector cells, promotion and enhancement of cell-mediated lysis, and direct complement-mediated cytolysis. The major inhibitors of complement activation on human cells are DAF and MCP. These proteins regulate complement enzymatic complexes that are involved in the amplification of the cascade and the resulting generation of C3/C4 opsonizing fragments and physiologically active C3a and C5a peptides. Formation of the cytolytic and proinflammatory MAC on host cell membranes is inhibited by CD59, a glycosylphosphatidyl inositol-linked glycoprotein that binds to C8 and C9 in the assembling complex.

Complement inhibitors have been found on nearly all primary tumors and cancer cell lines that have been examined, and some studies indicate that complement-inhibitory proteins are up-regulated on tumor cells. DAF and the serum complement inhibitor factor H or related proteins have been identified as tumor-associated antigens (1, 2), and the overexpression of DAF confers a poor prognosis in colorectal cancer patients (2). *In vitro* studies have shown that complement inhibitors expressed on tumor cells can inhibit both complement opsonization and direct cytolysis by the MAC (for recent reviews of immune evasion and complement resistance of tumor cells, see Refs. 3 and 4). However, there is little information regarding how tumor-expressed complement inhibitors relate to complement deposition *in situ*, and the *in vivo* relevance of complement effector mechanisms and the importance of tumor-expressed complement inhibitors in controlling tumor growth remain largely unexplored. One reason for this is that complement inhibitor proteins (particularly CD59) are species selective, and human complement inhibitors are less effective against rat and mouse complement (5, 6). Thus, endogenous complement inhibitors expressed on the surface of human tumor cells will not provide the cells with effective protection from complement in rodent models of human cancer. Indeed, the species-selective activity of membrane complement-inhibitory proteins may be a basis for observations that complement-activating mAbs effective at causing regression of human tumors in rodents have, in most cases, proven ineffective in clinical trials.

When investigating the role of complement-inhibitory proteins in immune evasion of tumor cells *in vivo*, it is therefore relevant to study rodent complement inhibitors in rodent models of cancer. The ubiquitous and high level of expression of membrane complement inhibitors on normal tissues has not allowed for the targeted blocking of complement inhibitors (using current technologies) on tumor cells in syngeneic rodent models of cancer. In the studies described here, we investigated the effect of heterologously expressed rat CD59 on the growth of a human neuroblastoma cell line in nude rats. The neuroblastoma cell line endogenously expressed CD59, but we have previously determined on a quantitative basis that human CD59 is severalfold less effective at inhibiting rat complement compared to human complement (6). The data show for the first time *in vivo* that the complement inhibitor CD59 expressed on a tumor cell surface significantly promotes tumor growth. We also show that growth *in vivo* resulted in the up-regulation of DAF on the tumor cell surface and that the level of DAF expression was further up-regulated by the expression of functional CD59.

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[2] To whom requests for reprints should be addressed, at Department of Microbiology & Immunology, Medical University of South Carolina, BSB 201, 173 Ashley Avenue, Charleston, SC 29425, E-mail: tomlinss@musc.edu.

[3] The abbreviations used are: MAC, membrane attack complex; DAF, decay-accelerating factor; MCP, membrane cofactor protein; mAb, monoclonal antibody.

MATERIALS AND METHODS

Cells and DNA. The LAN-1 neuroblastoma cell line was obtained from Dr. Robert Seeger (University of California at Los Angeles, Los Angeles, CA) and maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS (Hyclone, Logan, UT), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Incubation was at 37°C in 5% CO_2. cDNA encoding rat CD59 and cDNA encoding murine Ly6E were the gifts of Drs. B. P. Morgan (University of Wales, Cardiff, United Kingdom) and U. Haemmerling (Memorial Sloan-Kettering Cancer Center, New York, NY), respectively. Stably transfected LAN-1 cell populations were selected by fluorescence-activated cell sorting after the cultivation of cells in the presence of G418.

Antibodies and Complement. mAbs to human (YTH53.1) and rat (6D1) CD59 and rabbit antirat C9 polyclonal IgG were the gifts of Dr. B. P. Morgan. Human MCP mAb M75 (7) was a gift of Dr. D. M. Lublin (Washington University, St. Louis, MO). Antihuman DAF mAb 1A10 was described previously (8), and anti-GD2 3F8 mAb (9) was described previously. Goat antihuman C3 IgG cross-reactive with rat C3 was obtained from ICN Pharmaceuticals (Aurora, OH). Anti Ly6A/E mAb D7 was purchased from BD Pharmingen (San Diego, CA). FITC-conjugated antibodies used for flow cytometry were purchased from Sigma (St. Louis, MO). Normal human serum was obtained from the blood of healthy volunteers in the laboratory, and rat serum was obtained from the blood of normal and immune-deficient rats. Serum was stored in aliquots at −70°C until use.

Preparation of LAN-1 Transfectants. Rat CD59 cDNA and Ly6E cDNA were subcloned into the multiple cloning site of mammalian expression vector pCDNA3 (Invitrogen, Carlsbad, CA). DNA was transfected into 50–75% confluent LAN-1 cells using LipofectAMINE according to the manufacturer's instructions (Life Technologies, Inc., Grand Island, NY). Stable populations of LAN-1 cells expressing either rat CD59 or Ly6E were isolated by several rounds of cell sorting using either antirat CD59 mAb 6D1 or anti-Ly6A/E mAb D7 as described previously (10).

Complement Lysis Assays. Complement-mediated cell lysis was determined by both ^{51}Cr release (11) and enumeration after trypan blue staining (12), as described previously. Both methods gave similar results. Lysis assays of LAN-1 cells were performed using detached cells in both the absence and presence of antitumor complement-activating antibody. In assays in which cells were antibody-sensitized to complement, the anti-GD2 monoclonal antibody 3F8 was added at 15 μg/ml, and cells were incubated for 30 min at 4°C before the addition of rat serum. Experimental details have been described previously (13).

Flow Cytometric Analysis. Analysis of cell surface protein expression and complement protein deposition was performed by flow cytometry using appropriate antibodies (see above), as described previously (10). Primary antibodies and isotype-matched irrelevant control antibodies were used at a concentration of 10 μg/ml. Analysis was performed on cells removed from tissue culture using versene (Life Technologies, Inc.) for cell detachment and on cells isolated from excised tumors. Cell suspensions were obtained from tumors by gentle teasing of tumor tissue (in RPMI 1640/10% FCS) with scalpels, followed by low-speed centrifugation through Ficoll to remove tumor pieces and aggregates (14). Tumor-derived cells were then washed in RPMI 1640/10% FCS by centrifugation before use.

***In Vivo* Experiments.** Four-week-old male athymic *nu/nu* (nude) rats were obtained from the National Cancer Institute (Frederick, MD). The rats were housed in a clean room, and food and water were sterilized. Rats were injected s.c. in the right flank with the indicated numbers of LAN-1 cells suspended in 0.2 ml of PBS. Groups of rats received either LAN-1 cells transfected with rat CD59 or control-transfected LAN-1 cells. Control cells were transfected with Ly6E (a structural but not functional homologue of CD59) or with empty plasmid. There was no difference in tumor growth between the different control LAN-1 cells. Tumor volumes were calculated using the formula $4/3\pi r^3$ (volume of sphere). Statistical analyses were performed using the SAS system (SAS Institute Inc., Cary, NC).

RESULTS

Expression of Rat CD59 on LAN-1 Cells Confers Resistance to Rat Complement. We have previously shown that LAN-1 expresses CD59, DAF, and MCP and that the sensitivity of LAN-1 and LAN-1-derived clones to lysis by human complement can be significantly enhanced by blocking CD59 function. Blocking DAF function on LAN-1-derived clones only slightly enhanced sensitivity to human complement, whereas blocking MCP function had no effect (13). However, human CD59 is not an effective inhibitor of rat complement (6), and Fig. 1 shows that LAN-1 cells are significantly more sensitive to lysis by rat complement than lysis by human complement after sensitization by anti-GD2 3F8 mAb. LAN-1 cells express high levels of GD2 antigen, and the complement-activating properties of 3F8 mAb have been described previously (13, 15). Of note, LAN-1 cells are also lysed by rat complement in the absence of 3F8 mAb, albeit less effectively (Fig. 1*b*). These results confirm that endogenous expression of human complement inhibitors on LAN-1 cells does not provide effective protection from lysis by rat complement. Similar data were obtained with serum isolated from either normal or immune-deficient rats. Lysis of LAN-1 cells in the absence of sensitizing antibody may be due to the presence of natural endogenous complement-activating antibodies that bind to LAN-1 cells, and flow cytometric analysis of cells after incubation in heat-inactivated rat serum revealed that small amounts of rat immunoglobulin were deposited on the cell surface, supporting this possibility (data not shown).

LAN-1 cells were transfected with rat CD59, and LAN-1 cells stably expressing CD59 were isolated by cell sorting (Fig. 2). As a control for *in vivo* studies (see below), LAN-1 cells were also transfected with murine Ly6E antigen, a structural but not functional analogue of CD59, and sorted as described for rat CD59 transfectants. Fig. 3 shows that the expression of rat CD59 on LAN-1 cells significantly enhanced their resistance to lysis by rat complement, both in the absence and presence of complement-activating 3F8 mAb.

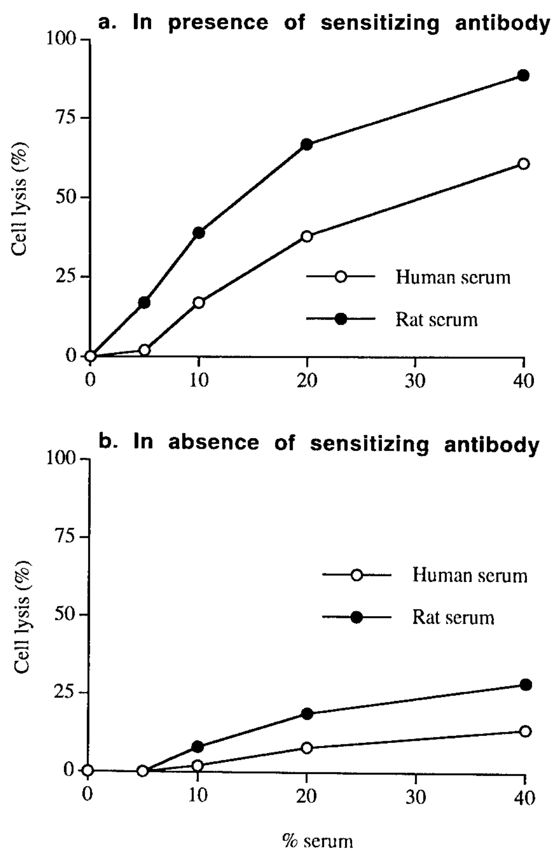

Fig. 1. Lysis of LAN-1 cells by human and rat complement. LAN-1 cells were incubated in the indicated concentration of rat or human serum in either the presence (*a*) or absence (*b*) of anti-GD2 complement-activating antibody (3F8 mAb). Complement-mediated cell lysis was determined after a 1-h incubation at 37°C. Representative data from at least three experiments are shown.

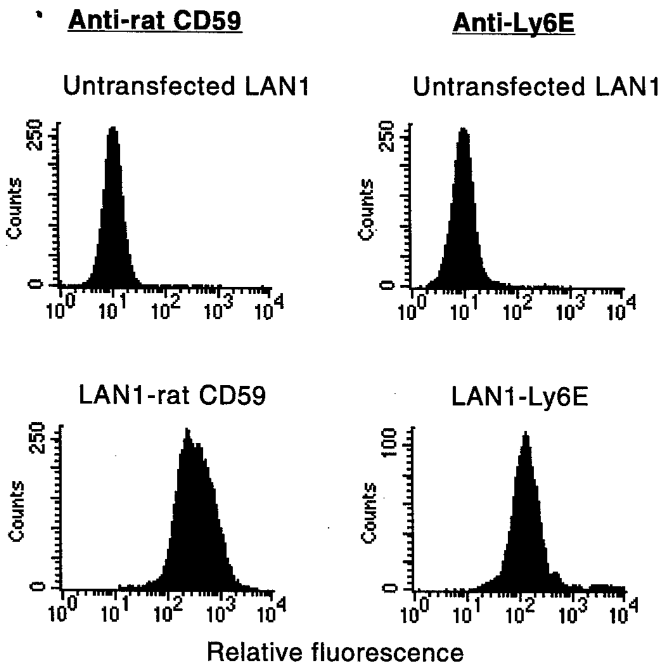

Fig. 2. Expression of rat CD59 and Ly6E on transfected LAN-1 cells. Stably transfected homogenous populations of LAN-1 cells expressing rat CD59 or murine Ly6E were isolated by several rounds of cell sorting. The figure shows flow cytometric analysis of sorted populations. Cells were stained by immunofluorescence using antirat CD59 mAb (6D1) or anti-Ly6E mAb (D7). Histograms of the relative fluorescence intensities are shown.

Expression of Rat CD59 on LAN-1 Enhances Tumorigenicity in Nude Rats. We first determined the tumorigenicity of LAN-1 cells in immune-deficient rats. The result of a dose-response experiment after s.c. injection of LAN-1 cells into the flank of nude rats is shown in Table 1. To investigate the effect of CD59 expression and increased complement resistance on *in vivo* tumor growth, control-transfected LAN-1 cells and LAN-1 cells stably expressing rat CD59 were injected separately into nude rats, and tumor growth was monitored. Groups of nude rats were inoculated with either 8×10^6 cells, a dose resulting in almost 100% tumor take for untransfected LAN-1 cells, or 4×10^6 cells, a dose determined to result in tumor growth in approximately 50% of animals (Table 1).

When LAN-1 cells expressing rat CD59 were injected into nude rats at a dose of 4×10^6, 100% of rats grew tumors, and the onset of tumor growth was earlier than that seen for control-transfected LAN-1 cells ($P < 0.01$, χ^2 analysis). Regression analysis showed that the rate of tumor growth was also significantly faster in rats inoculated with rat CD59-transfected cells ($P < 0.01$). In addition, analysis of the mean difference in tumor size on each day of tumor measurement between the two groups of rats showed that tumors growing in rats inoculated with rat CD59-transfected cells were significantly larger, with Ps < 0.01 and an average P value of 0.0021 (Student's t test; Fig. 4*a*).

Increasing the inoculation dose to 8×10^6 cells resulted in almost 100% tumor take with both rat CD59-transfected cells (19 of 19 rats) and control cells (19 of 21 rats), as expected from the dose-response data shown in Table 1. The onset of tumor growth, however, occurred significantly earlier in rats inoculated with rat CD59-transfected cells (Fig. 5); the mean day of tumor onset was day 13 for rats inoculated with control LAN-1 cells and day 7.4 for rats inoculated with rat CD59-transfected LAN-1 cells. One week after inoculation, 7 of 21 rats inoculated with control LAN-1 cells contained tumors, whereas 15 of 19 rats inoculated with rat CD59-transfected LAN-1 cells contained tumors. This is a highly significant difference ($P = 0.001$, χ^2 analysis). Similar to the data obtained with an inoculum of 4×10^6 cells, there was also a highly significant difference in the mean tumor size between rats inoculated with either control or rat CD59-transfectants at each day of tumor measurement, with Ps < 0.01 and an average $P < 0.001$ (Student's t test; Fig. 4*b*).

Although there was a highly significant difference in the rate of tumor growth between rat CD59- and control-transfected LAN-1 cells when rats were inoculated with 4×10^6 cells, there was a less pronounced difference in rats inoculated with a higher number of cells (compare Fig. 4, *a* and *b*). In this context, our data indicate the presence of low concentrations of natural endogenous antibodies in nude rats that bind to LAN-1 cells (see above), and when a high cell inoculum or after a threshold tumor size is reached, it is possible that endogenous antitumor antibodies may become depleted. At this point, complement may no longer be effectively activated at the tumor cell surface, and complement-sensitive (control-transfected cells) and -resistant cells (rat CD59-transfected cells) may grow at similar rates.

Complement Deposition and Expression of Complement Inhibitors on Tumor-derived LAN-1 Cells. Cells isolated from tumors after 28 days of growth were initially analyzed for deposition of

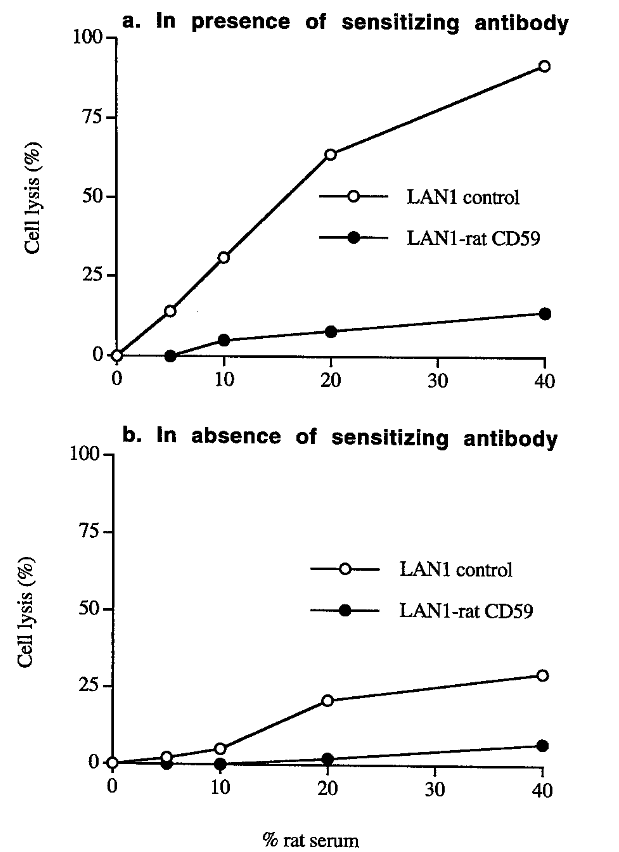

Fig. 3. Rat complement-mediated lysis of LAN-1 cells and LAN-1 cells expressing rat CD59. LAN-1 cells or LAN-1 cells stably expressing rat CD59 were incubated in the indicated concentration of rat serum in either the presence (*a*) or absence (*b*) of anti-GD2 complement-activating antibody (3F8 mAb). Complement-mediated cell lysis was determined after a 1-h incubation at 37°C.

Table 1 *Tumor incidence of LAN-1 cells in immune-deficient rats*

No. of cells injected[a]	No. of rats with tumor/ no. of rats inoculated
1×10^7	8/8
8×10^6	9/10
4×10^6	4/10
1×10^6	0/6

[a] Rats were inoculated s.c. in the flank and examined for tumor growth for up to 30 days.

complement and the continued expression of transfected rat CD59 by flow cytometry. As shown in Fig. 6, expression of rat CD59 was maintained on the tumor cells at a level similar to that seen in *in vitro* cultured cells used for inoculation. Interestingly, the level of Ly6E expression on control-transfected LAN-1 cells was not maintained during *in vivo* growth. This finding may be the result of selective pressure exerted by rat complement on rat CD59 expression.

As shown above (see Fig. 1), unsensitized LAN-1 cells are lysed by rat complement *in vitro*, and, as anticipated, complement proteins C3 and C9 were both deposited on LAN-1 tumors *in vivo*. Less deposited C9 was detected on rat CD59-transfected tumor-derived cells than on tumor-derived control LAN-1 cells (Fig. 6), consistent with the known function of CD59. More surprising was the finding that rat CD59-transfected tumor cells also had lower levels of C3 deposited on their surface as compared with control cells; the difference was small but consistent (Fig. 6 shows the results from a representative analysis). This was surprising because CD59 does not inhibit complement activation and is not expected to influence C3 deposition. An explanation for these data was provided, however, when we analyzed the endogenous expression of complement inhibitors on LAN-1 cells. We compared the relative levels of endogenously expressed DAF, MCP, and CD59 between *in vitro* cultured LAN-1 cells and LAN-1 cells isolated from tumors. Fig. 6 shows that DAF expression was up-regulated on the surface of tumor-derived control LAN-1 cells by about twofold compared with *in vitro* cultured cells. The relative level of DAF expressed on rat CD59-transfected cells derived from tumors was even further up-regulated compared with that in cells grown *in vitro* (about threefold). Thus, the increased level of DAF expression is likely to account for the decreased level of C3 deposited on the rat CD59-transfected tumor-derived cells. Of relevance to this finding, human DAF is known to inhibit rat complement, albeit less effectively than human complement (see "Discussion"). Multiple tumors from separate experiments were analyzed by flow cytometry, and the data shown in Fig. 6 are representative of at least six determinations for particular antigen groups. Transfection of LAN-1 with rat CD59 did not alter the level of endogenous DAF expression on cells cultured *in vitro*, and the level of endogenous CD59 and MCP expression on LAN-1 cells was unchanged after *in vivo* growth (Fig. 6). It is unlikely that the increased levels of DAF on LAN-1 cells after *in vivo* growth are due to selection because populations expressing higher-than-normal amounts of DAF could not be selected by cell sorting *in vitro*, and selection is not consistent with the finding that even higher levels of DAF are seen on rat CD59-expressing cells grown *in vivo*.

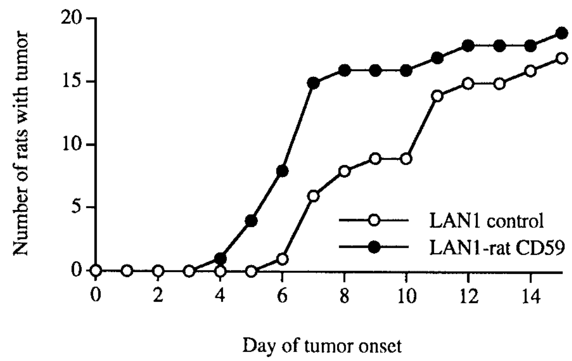

Fig. 5. Effect of rat CD59 expression on the onset of LAN-1 tumor growth. Control LAN-1 or rat CD59-transfected LAN-1 cells (8×10^6) were injected s.c. into the flank of nude rats, and the rats were examined daily for the appearance of a tumor (tumor recorded at a minimum diameter of 0.25 cm). $n = 21$ for the control group, and $n = 19$ for the rat CD59-transfected group.

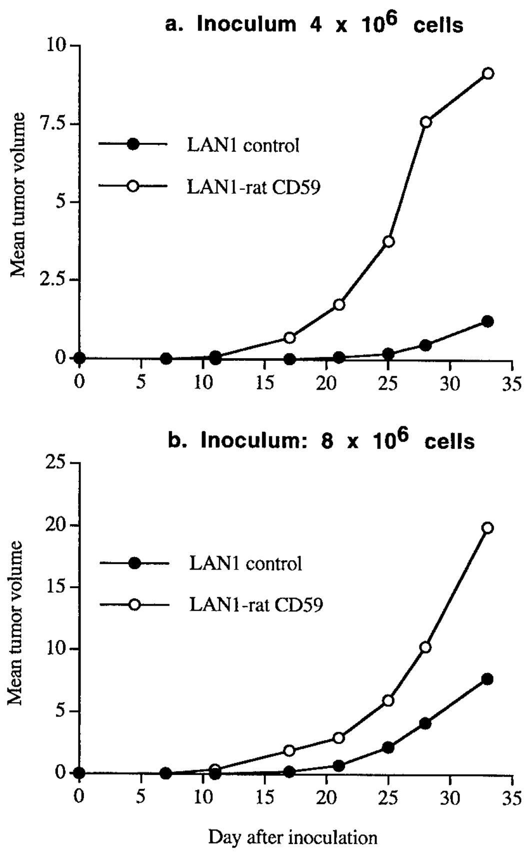

Fig. 4. Growth curves of control LAN-1 cells and rat CD59-transfected LAN-1 cells in nude rats. Either 4×10^6 cells (*a*) or 8×10^6 cells (*b*) were injected s.c. into the flank of nude rats. Growth was measured at intervals for 33 days. For experiment with a 4×10^6 inoculum (*a*), $n = 8$ rats/group; for the 8×10^6 inoculum (*b*), $n = 19$ rats/group.

DISCUSSION

It has been hypothesized that complement inhibitors on the surface of tumor cells present a barrier to immune-mediated clearance of tumor cells by contributing to the ineffectiveness of humoral immune responses observed in some cancers or by preventing effective mAb-mediated immunotherapy. Nearly all human tumor cells examined express membrane complement-inhibitory proteins, and most display a high level of resistance to lysis by human complement *in vitro*, even in the presence of antitumor complement-activating antibodies. On the other hand, human tumor cell lines are more susceptible to lysis by heterologous complement. We show here that the LAN-1 human neuroblastoma cell line is highly susceptible to lysis by rat complement, despite the endogenous expression of complement-inhibitory proteins. Of relevance to this finding, we have shown previously that human CD59 is not an effective inhibitor of rat complement (6). Here, we established a LAN-1 neuroblastoma cell line stably expressing rat CD59 for use in a rat model of human cancer relevant for studying the role of complement and complement inhibitors. Using this model, we demonstrate directly that a complement inhibitor expressed on the surface of a tumor cell can influence tumor growth. We also found that DAF was up-regulated at the LAN-1 tumor cell surface after growth *in vivo* and that DAF was even further up-regulated on tumor cells expressing functional (rat) CD59 when grown *in vivo*. Increased DAF expression was associated with decreased C3 deposition. These data demonstrate that the expression of complement inhibitors on a tumor cell has functional consequences with regard to complement deposition and tumor growth.

The expression of membrane-bound complement-inhibitory pro-

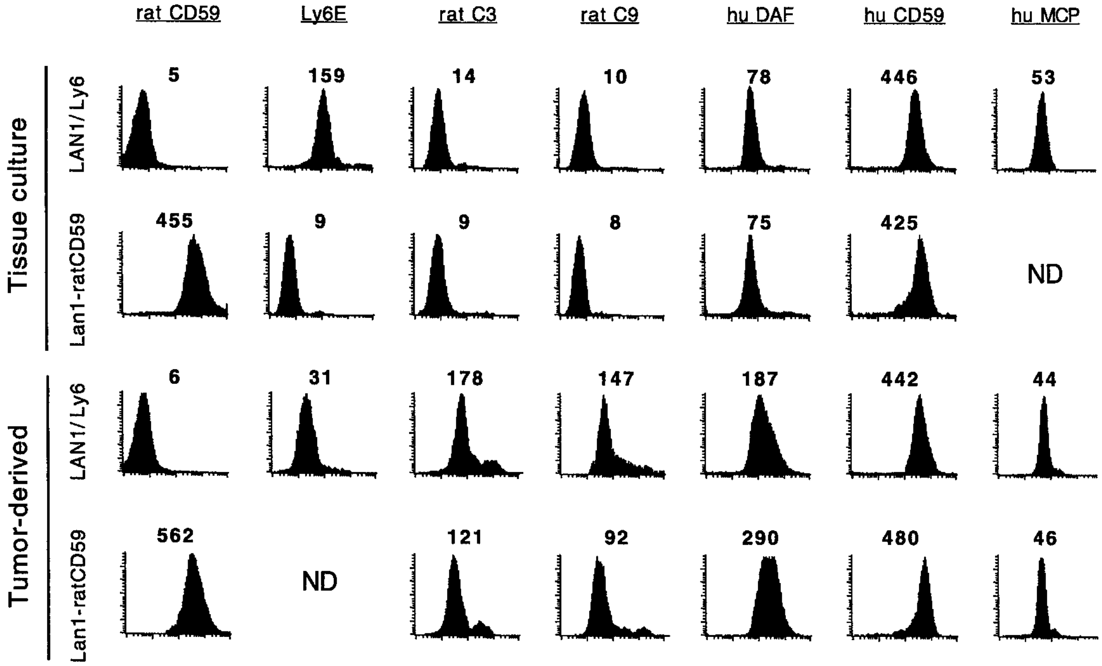

Fig. 6. Flow cytometric analysis of LAN-1 and rat CD59-transfected LAN-1 cells. Control or rat CD59-transfected LAN-1 cells grown in tissue culture (*top two rows*) or cells isolated from tumors (*bottom two rows*) were analyzed for expression of complement inhibitors and for the deposition of complement proteins as indicated. Cells were stained by immunofluorescence using appropriate antibodies (see "Materials and Methods"). The figure shows histograms of relative fluorescence, with *numerals* indicating the relative mean fluorescence intensities. Representative data are shown from at least six separate analyses for each antigen.

teins may benefit tumor cells for several reasons. Complement activation products (particularly C5a and the MAC) are powerful mediators of inflammation and may promote the recruitment of immune effector cells to the site of tumor growth. Cell-bound C3 activation products can promote and enhance antibody-dependent cell cytotoxicity and natural killer effector systems, and formation of the MAC can be directly cytolytic. Therefore, at least conceptually, it is reasonable to consider that up-regulation of complement inhibitors, as we observe here for DAF, may represent a mechanism by which some tumors can escape immune destruction. DAF is an inhibitor of complement activation and will inhibit the generation of C3/C5 activation products as well as the terminal MAC, whereas CD59 inhibits only MAC assembly. Because of the effect of CD59 on DAF expression, the current data do not provide information on the relative roles that these mechanisms may play in controlling tumor growth. However, the data do clearly establish that complement is involved in controlling tumor growth in this model and that CD59 promotes tumor growth whichever complement-associated mechanism(s) is operative.

So how does *in vivo* growth and, in particular, the expression of functional CD59 modulate DAF expression? Complement activation products and various cytokines have been reported to modulate complement inhibitor expression *in vitro*, although the effects appear to be variable, particularly for DAF (3, 4, 16–21). Also, a recent *in vitro* study reported that assembly of the MAC on endothelial cells directly up-regulated DAF expression and that expression was enhanced by cytokines (16). Similar mechanisms may be responsible for the up-regulation of DAF on tumor cells *in vivo*, as reported here. To explain the higher levels of DAF observed on rat CD59-expressing LAN-1 cells derived from tumors, it is conceivable that CD59-expressing cells may be able to survive higher levels of MAC that are initially deposited on the cell surface, thus enhancing the signal for DAF expression. CD59 limits the number of C9 molecules bound per MAC, and complexes containing bound C9, but with abrogated lytic function, may still be able to provide the signal for DAF up-regulation. It is also possible that the signal for induction of DAF expression is delivered via rat CD59 after its engagement by assembling complement complexes. This notion is consistent with the demonstration that CD59 is a signal transducing molecule (22–26). Increased endogenous DAF expression on LAN-1 cells correlated with decreased rat C3 deposition, and in this regard, human DAF is able to inhibit rat complement, although it is a less effective inhibitor of rat complement than human complement.[4]

We show that LAN-1 cells activate rat complement in the absence of exogenously added complement-activating antibody both *in vitro* and *in vivo*. This is probably due to the presence of natural endogenous xenogeneic antibodies because rat immunoglobulin is deposited on the LAN-1 cell surface after the incubation of cells in nude rat serum. It may be that tumor cell lines that do not "spontaneously" activate rodent complement will require the administration of exogenous complement-activating antitumor antibodies for an effect of complement inhibitors on tumor growth to be observed in rodent hosts. Indeed, human tumor cell lines transfected with rodent complement inhibitors and grown in rodents may represent good preclinical models relevant for evaluating tumor-specific mAbs. For our studies, we chose to use a rat model because the rat complement system appears to be more robust than the murine complement system and may represent a better model. It is difficult to isolate hemolytically active mouse complement, and there are reports documenting low complement levels in common laboratory mouse strains and nude mouse strains as compared with complement levels found in humans and rats (27, 28).

In summary, our results show that a membrane complement inhibitor expressed on the surface of a tumor cell plays a role in determining tumorigenesis and that reversing the effects of tumor-specific complement regulators is likely to enhance immune-mediated clear-

[4] C. L. Harris, O. B. Spiller, and B. P. Morgan, personal communication.

ance of some tumors. The widespread expression of membrane-bound complement inhibitors presents technical difficulties for the selective blocking of complement inhibitors on tumor cells. However, it may be possible to adapt current and developing technologies to permit targeted delivery of antibodies, peptides, or perhaps antisense DNA to block the effects of endogenous complement inhibitors expressed on tumor cells.

REFERENCES

1. Kinders, R. J., Jones, T., Root, R., Bruce, C., Murchison, H., Corey, M., Williams, L., Enfield, D., and Hass, G. M. Complement factor H or a related protein is a marker for transitional cell cancer of the bladder. Clin. Cancer Res., *4:* 2511–2520, 1998.
2. Spendlove, I., Li, L., Carmichael, J., and Durrant, L. G. Decay-accelerating factor (CD55): a target for cancer vaccines. Cancer Res., *59:* 2282–2286, 1999.
3. Maio, M., Brasoveanu, L. I., Coral, S., Sigalotti, L., Lamaj, E., Gasparollo, A., Visintin, A., Altomonte, M., and Fonsatti, E. Structure, distribution, and functional role of protectin (CD59) in complement-susceptibility and immunotherapy of human malignancies. Int. J. Oncol., *13:* 305–318, 1998.
4. Gorter, A., and Meri, S. Immune evasion of tumor cells using membrane-bound complement regulatory proteins. Immunol. Today, *20:* 576–582, 1999.
5. Yu, J., Caragine, T., Chen, S., Morgan, B. P., Frey, A. F., and Tomlinson, S. Protection of human breast cancer cells from complement-mediated lysis by expression of heterologous CD59. Clin. Exp. Immunol., *115:* 13–18, 1999.
6. Yu, J., Dong, S., Rushmere, N. K., Morgan, B. P., Abagyan, R., and Tomlinson, S. Mapping the regions of the complement inhibitor CD59 responsible for its species selectivity. Biochemistry, *36:* 9423–9428, 1997.
7. Seya, T., Hara, T., Matsumoto, M., and Akedo, H. Quantitative analysis of membrane cofactor protein (MCP) of complement. J. Immunol., *145:* 238–245, 1990.
8. Kinoshita, T., Medof, M. E., Silber, R., and Nussenzweig, V. Distribution of decay-accelerating factor in peripheral blood of normal individuals and patients with paroxysmal nocturnal hemoglobinuria. J. Exp. Med., *162:* 75–92, 1985.
9. Cheung, N-K. V., Saavinen, U. M., Neely, J. E., Landmeier, B., Donovan, D., and Coocia, P. F. Monoclonal antibodies to a glycolipid antigen on human neuroblastoma cells. Cancer Res., *45:* 2642–2650, 1985.
10. Yu, J., Abagyan, R. A., Dong, S., Gilbert, A., Nussenzweig, V., and Tomlinson, S. Mapping the active site of CD59. J. Exp. Med., *185:* 745–753, 1997.
11. Helfand, S. C., Hank, J. A., Gan, J., and Sondel, P. M. Lysis of human tumor cell lines by canine complement plus monoclonal antiganglioside antibodies or natural canine xenoantibodies. Cell. Immunol., *167:* 99–107, 1996.
12. Rushmere, N. K., Tomlinson, S., and Morgan, B. P. Expression of rat CD59: functional analysis confirms lack of species specificity and reveals that glycosylation is not required for function. Immunology, *90:* 640–646, 1997.
13. Chen, S., Caragine, T., Cheung, N. K. V., and Tomlinson, S. Surface antigen expression and complement susceptibility of differentiated neuroblastoma clones. Am. J. Pathol., *156:* 1085–1091, 2000.
14. Wilson, A. P. Preparation of tumor cell lines. *In:* G. Gallagher, R. C. Rees, and C. W. Reynolds (eds.), Tumor Immunology: A Practical Approach, pp. 1–14. Oxford, United Kingdom: Oxford University Press, 1993.
15. Saarinen, U. M., Coccia, P. F., Gerson, S. L., Pelley, R., and Cheung, N. K. V. Eradication of neuroblastoma cells *in vitro* by monoclonal antibody and human complement: method for purging autologous bone marrow. Cancer Res., *45:* 499–503, 1985.
16. Mason, J. C., Yarwood, H., Sugars, K., Morgan, B. P., Davies, K. A., and Haskard, D. O. Induction of decay accelerating factor by cytokines or the membrane attack complex protects vascular endothelial cells against complement deposition. Blood, *94:* 1673–1682, 1999.
17. Bjorge, L., Jensen, T. S., and Matre, R. Characterization of the complement regulatory proteins decay accelerating factor (DAF, CD55) and membrane cofactor protein (MCP, CD46) on a human colonic adenocarcinoma cell line. Cancer Immunol. Immunother., *42:* 185–192, 1996.
18. Varsano, S., Rashkovsky, L., Shapiro, H., and Radney, J. Cytokines modulate expression of cell-membrane complement inhibitory proteins in human lung cancer cell lines. Am. J. Respir. Cell Mol. Biol., *19:* 522–529, 1998.
19. Cosio, F. G., Shibata, T., Rovin, B. H., and Birmingham, D. J. Effects of complement activation products on the synthesis of decay accelerating factor and membrane cofactor protein by human mesangial cells. Kidney Int., *46:* 986–992, 1994.
20. Berger, M., and Medof, M. E. Increased expression of complement decay accelerating factor during activation of human neutrophils. J. Clin. Invest., *79:* 214–220, 1987.
21. Bjorge, L., Jensen, T. S., Ulvestad, E., Vedeler, C. A., and Matre, R. The influence of tumour necrosis factor-α, interleukin-1β and interferon-γ on the expression and function of the complement regulatory protein CD59 on the human colonic adenocarcinoma cell line HT29. Scand. J. Immunol., *41:* 350–356, 1995.
22. Korty, P. E., Brando, C., and Shevach, E. M. CD59 functions as a signal-transducing molecule for human T cell activation. J. Immunol., *146:* 4092–4098, 1991.
23. Hahn, W. C., Menu, E., Bothwell, A. L., Sims, P. J., and Bierer, B. E. Overlapping but nonidentical binding sites on CD2 for CD58 and a second ligand CD59. Science (Washington DC), *256:* 1805–1807, 1992.
24. Deckert, M., Kubar, J., Zoccola, D., Bernard-Pomier, G., Angelisova, P., Horejsi, V., and Bernard, A. CD59 molecule: a second ligand for CD2 in T cell adhesion. Eur. J. Immunol., *22:* 2943–2947, 1992.
25. Morgan, B. P., Van den Berg, C. W., Davies, E. V., Hallett, M. B., and Horejsi, V. Cross-linking of CD59 and of other glycosyl phosphatidylinositol-anchored molecules on neutrophils triggers cell activation via tyrosine kinase. Eur. J. Immunol., *23:* 2841–2850, 1993.
26. van den Berg, C., Cinek, T., Hallett, M. B., Horejsi, V., and Morgan, B. P. Exogenous glycosyl phosphatidylinositol-anchored CD59 associates with kinases in membrane clusters on U937 cells and becomes Ca^{2+}-signalling. J. Cell. Biol., *131:* 669–677, 1995.
27. Ong, G. L., and Mattes, M. J. Mouse strains with typical mammalian levels of complement activity. J. Immunol. Methods, *125:* 147–158, 1989.
28. Ish, C., Ong, G. L., Desai, N., and Mattes, M. J. The specificity of alternative complement pathway-mediated lysis of erythrocytes: a survey of complement and target cells from 25 species. Scand. J. Immunol., *38:* 113–122, 1993.

A Tumor-Expressed Inhibitor of the Early but not Late Complement Pathway Enhances Tumor Growth in a Rat Model of Human Breast Cancer

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The abbreviations used are: DAF, decay accelerating factor; FCS, fetal calf serum; mAb, monoclonal antibody; MAC, membrane attack complex; NHS, normal human serum

Abstract

Membrane-bound complement inhibitors protect host cells from inadvertent complement attack and complement inhibitors are often upregulated on tumors, possibly representing a selective adaptation by tumors to escape elimination by a host anti-tumor immune response. Relevant in vivo studies using rodent models of human cancer have been hampered by the fact that human complement inhibitors are not effective against rodent complement. Using nude rats and MCF7 cells expressing different rat complement inhibitors, a model of human breast cancer was established to investigate the role of complement and complement inhibitors in tumor progression. Expression of rat CD59, an inhibitor of the terminal cytolytic membrane attack complex (MAC) of complement, had no effect on the incidence or growth rate of MCF7 tumors. In contrast, expression of rat Crry, an inhibitor of complement activation, dramatically enhanced the tumorigenicity of MCF7 cells. The expression of rat Crry on MCF7 inhibited the in vivo deposition of complement C3 fragments that serve as opsonins for receptors on phagocytes and NK cells. These data provide direct in vivo evidence that an inhibitor of complement activation can facilitate tumor growth by modulating C3 deposition. This observation demonstrates an important role for complement opsonization in promoting cell-mediated anti-tumor immune function. Rat complement activation on MCF7 tumors was mediated by tumor reactive antibodies present in the serum of naïve nude rats, but there was also an IgM response to MCF7 tumors, a situation with similarities to some human cancers. These data support a hypothesis that blocking complement inhibitor function on tumor cells will not only enhance monoclonal antibody-mediated immunotherapy, but may also be effective at enhancing a normally ineffective humoral immune response in the absence of administered anti-tumor antibody.

Introduction

Complement activation on a cell surface results in the formation of cell-bound C3/C5 convertases, enzyme complexes that cleave serum complement proteins C3 and C5. Cleaved C3 fragments can become covalently bound to the activating cell surface where they can amplify the complement cascade and can serve as opsonins for immune effector cells. C3 opsonization can be important for promoting and enhancing antibody-dependent cell cytotoxicity (ADCC) and natural killer (NK) cell-mediated lysis, effector systems that are believed to be important in immune resistance to tumors. It was recently shown that inhibitory Fc receptors can modulate in vivo cytoxicity, confirming the physiological significance of ADCC in an anti-tumor immune response(1). Cleavage of C5 yields C5a and C5b fragments. C5a is a chemoattractant and a powerful mediator of inflammation that may potentiate anti-tumor responses. C5b initiates the sequential recruitment of the terminal complement proteins (C6, C7, C8 and C9) to form the membrane attack complex (MAC) on the activating cell surface. If deposited on a tumor cell in sufficient quantity, the MAC is directly cytolytic, but when deposited in sublytic concentrations the MAC can also stimulate cells to release proinflammatory molecules. Complement activation on a tumor cell surface can result from the binding of exogenously administered tumor reactive antibodies (immunotherapy). In addition, a humoral immune response to tumors has been described and there are reports of persisitent complement activation on breast (2) and other tumor cells (3, 4).

Both normal and tumor cells are protected from complement attack by different membrane-bound complement inhibitory proteins. In humans, complement *activation* is controlled primarily by the membrane proteins decay-accelerating factor (DAF) and membrane cofactor protein (MCP). A complement activation inhibitor termed Crry is expressed in rodents, but not humans. Although rodent cells also express DAF and MCP, the expression of Crry is broader and Crry is considered the rodent functional and structural analogue of human DAF and MCP (5-7). These regulators of complement activation act early in the pathway and inhibit the generation of C3 opsonins and the soluble inflammatory activation fragments C3a and C5a, and may thus modulate a cell-mediated anti-tumor effector response. Control of cytolytic MAC formation on cell membranes is provided by CD59, a cell surface glycoprotein that binds to the terminal proteins in the complement cascade (C8 and C9) and prevents their assembly into a lytic complex (8, 9). Therefore, CD59 has no direct effect on the generation of C3 opsonins and C3/C5 peptide activation products, but has the potential to modulate direct complement-mediated lysis of tumor cells.

Complement inhibitors have been identified as tumor associated antigens (10, 11) and their expression is upregulated on some tumor cells. There is good in vitro evidence to support the hypothesis that complement inhibitors expressed on tumor cells can provide protection from immune-surveillance and that they present a barrier to successful antibody-mediated immunotherapy (for reviews on the subject, see (12-14)). However, there is a paucity of in vivo data to support these hypotheses, one reason being the species selectivity of complement inhibitors and the lack of a suitable rodent model. Here we report the development of a clinically relevant nude rat model of human breast cancer and its use to study the role of different types of tumor-expressed complement inhibitors on tumor progression.

Materials and Methods

Cell lines. The human breast cancer cell line MCF7 (American Type Culture Collection, Rockville, MD) and MCF7 transfectants were grown at 37°C in 5% $C0_2$ in Eagle's modified essential medium (EMEM) supplemented with 10% heat-inactivated FCS (Hyclone, Logan, Utah), 0.1% non-essential amino acids, human insulin (10 ug/ml) and 2mM glutamine. For preparation of transfected MCF7 cell lines, cDNA encoding rat CD59 (15) and rat Crry (6) was subcloned into the mammalian expression vector pCDNA3 and pCDNA3.1 (Invitrogen, Carlsbad, CA), respectively, and transfected into MCF7 cells using Lipofectamine plus (Gibco BRL, Grand Island, NY) according to the manufacturer's instructions. Stably transfected MCF7 cells were selected following the cultivation of cells in the presence of G418 (PCDNA3) or zeocin (PCDNA3.1), and populations expressing uniform levels of each rat complement inhibitor were isolated by several rounds of cell sorting (16). Double rat CD59/Crry transfectants were prepared by transfecting stable Crry transfectants with rat CD59.

Antibodies and serum. Rabbit antisera to MCF7 cell membranes that was used to sensitize MCF7 cells to complement for in vitro assays was prepared by standard techniques (17). Anti-rat Crry mAb 5I2 was described previously (18). Anti-rat CD59 mAb 6D1 and anti-MUC1 mAb C595 were provided by Drs. B. P. Morgan (University of Wales, Cardiff, U.K.) and M. R. Price (University of Nottingham, U.K.) respectively. FITC-labeled goat anti-human C3 (cross-reactive with rat C3) was purchased from ICN Pharmaceuticals (Aurora, OH). Antibodies to rat Ig and secondary FITC-labeled antibodies for flow cytometry were purchased from Sigma (St. Louis, MO). Rat serum was prepared from blood collected by heart puncture, and serum processed after clotting for three hours on ice. C6-deficient rat serum was the kind gift of Dr. W. M. Baldwin (Johns Hopkins University School of Medicine, Baltimore, MD).

Complement lysis assay. Rat complement-mediated lysis was determined using cells either in the presence or absence of anti-MCF7 complement activating antibodies. For antibody sensitization, cells were incubated in 10% anti-MCF7 antiserum for 30 min. on ice, washed, and resuspended in assay medium. Cell lysis was determined by ^{51}Cr release as previously described (19).

Flow cytometry. Analysis of cell surface expression of rat complement inhibitors on transfected MCF7 cells was performed using appropriate antibodies as described (16). For analysis of rat C3 deposition on MCF7 cells in vitro, versene detached cells were first incubated in different concentrations of anti-MCF7 antiserum as described above for lysis assays. Washed cells were then incubated in 40% C6-deficient rat serum (to prevent MAC-mediated lysis) for 30 min/37°C, washed, fixed with 2% paraformaldehyde and analyzed for deposited C3 by means of FITC-conjugated anti-rat C3. For analysis of rat IgG and IgM binding to tissue culture-derived MCF7, cells were incubated in 40% heat inactivated serum (56°C/30 min) isolated from either naïve or tumor-bearing rats. Following 60 min incubation on ice, cells were washed, fixed and stained with appropriate FITC-labeled secondary antibody. For analysis of tumor-derived cells, cell suspensions were prepared by gentle teasing of isolated tumor tissue with scalpels (in RPMI/10% FCS) followed by centrifugation through Ficoll to remove tumor pieces and aggregates. Isolated cells were then washed by centrifugation in RPMI/10% FCS before use. Viability was checked by trypan blue exclusion. Procedure for staining for flow cytometry was previously described (16). In all assays, control experiments were performed using isotype matched antibodies of irrelevant specificity.

Rat tumor model. Four-week-old female athymic nu/nu (nude) rats were obtained from the National Cancer Institute (Frederick, MD). Rats were housed in a clean room, and food and water was sterilized. Five mg 90 day release 17β–estradiol pellets (Innovative Research, Saratoga Springs, FL) were implanted with a trocar between scapulae of anesthetized rats. Five days later, rats were injected in left ventral mammary pad with 5 x 10^6 cells suspended in 0.2 ml PBS. Groups of rats received MCF7 cells transfected with either rat CD59, rat Crry, rat CD59+rat Crry, or mock-transfected with empty plasmid. In one experiment (not shown) control cells were transfected with mouse Ly6E (a structural but not functional homologue of CD59), and these cells exhibited the same growth characteristics as mock-transfected MCF7. Tumor growth was detected typically between 20-30 days. Tumor diameters were measured with vernier calipers and volume calculated from formula $4/3\pi r^3$. Statistical analyses were performed using the intrinsic function of Student's T test within Excel (Microsoft, Redmond, WA).

Results

Expression of rat complement inhibitors on the surface of human MCF7 cells confers resistance to rat complement in vitro. Endogenous expression of CD59 and DAF provides human MCF7 cells with resistance to lysis mediated by human complement, even in the presence of complement activating anti-MCF7 antibody (19, 20). However, MCF7 cells are susceptible to lysis by rat complement, illustrating the species selectivity of human complement inhibitors (19). Therefore, to study the effects of complement inhibitors in a rodent model, MCF7 were transfected with either rat Crry, rat CD59 or both, and stably expressing populations isolated (fig. 1). MCF7 cells expressing rat CD59 or Crry were highly resistant to rat complement-mediated lysis (fig. 2). Rat Crry transfected cells, but not rat CD59 transfected cells were also resistant to rat complement C3 deposition (fig. 3). When measuring C3 deposition, C6-deficient rat serum was used to prevent cytolytic MAC formation. The resistance of the rat CD59 and Crry transfected MCF7 cells to rat complement parallels the resistance of MCF7 cells to human complement.

Crry, but not CD59 promotes tumor growth in nude rats. To investigate the effect of rat complement inhibitors on the growth of MCF7 tumors in rats, nude rats were inoculated subcutaneously with 5×10^6 transfected or mock transfected MCF7 cells and tumor growth monitored . This inoculum was below the previously determined minumum tumorigenic dose (50% tumor take) of 1×10^7 cells. As expected, only 15% of rats inoculated with mock transfected MCF7 grew tumors (fig. 4). Similarly, less than 15% of rats inoculated with rat CD59 transfected MCF7 developed tumors (fig. 4). In sharp contrast, however, 100% of rats inoculated with MCF7 cells expressing rat Crry, either alone or together with CD59, developed tumors (fig. 4). There was also a dramatic difference in the rate of tumor growth in rats inoculated with Crry expressing MCF7 cells compared to rats inoculated with either mock or rat CD59 transfected MCF7 cells (fig. 5), although the incidence of tumors was low with mock and rat CD59 transfected cells. The expression of rat CD59 on MCF7 cells did not enhance tumor growth, but its coexpression with rat Crry on MCF7 further enhanced the effect of Crry on tumor growth; analysis of the mean difference in tumor size on each day of tumor measurement shows a statistically significant increase in tumor size for rat CD59+Crry transfectants compared to Crry transfectants ($P<0.01$) (fig. 5).

Analysis of tumor-derived MCF7 cells. Transfected tumor-derived MCF7 cells continued to express rat complement inhibitory proteins (fig. 6). When compared to cells used for inoculation, there was no change in the expression levels of rat CD59 or rat Crry in either single or double transfectants following in vivo growth. Tumor-derived MCF7 cells, whether mock transfected (fig. 6) or transfected with rat complement inhibitors (not shown) also continued to express the tumor-associated antigen MUC1 on their surface. We had previously found that in vivo growth of a human neuroblastoma cell line in rats resulted in an upregulation of endogenously expressed (human) DAF (21), but we found no upregulation of any of the endogenously expressed complement inhibitors (DAF, MCP or CD59) on MCF7 following in vivo growth (not shown). To determine whether the expression of Crry on MCF7 has functional consequences with regard to complement deposition in vivo, tumor-derived MCF7 cells were examined for deposited C3. There was a negative correlation between Crry expression and C3 deposition, with deposited C3 being detected only on mock transfected MCF7 cells. No C3 was detected on Crry expressing MCF7 cells (fig.7). Similar data was obtained when control and transfected MCF7 cells were incubated in nude rat serum in vitro (see fig. 3).

To investigate the mechanism of in vivo complement activation by MCF7 cells, tumor-derived MCF7 cells were analyzed for the deposition of rat antibody. By flow cytometry moderate amounts of bound IgM and lower levels of bound IgG were detected (fig. 6). To determine if there was an antibody response to MCF7 (presumably T-cell independent), tissue culture derived MCF7 cells were incubated with heat inactivated serum from naïve or MCF7 tumor-bearing nude rats, and antibody deposition analyzed. There was evidence of an IgM, but not an IgG response (fig. 8). There was no difference between antibody binding to either mock transfected or transfected MCF7 cells (not shown). IgM is an effective activator of complement, and in correlation with increased anti-MCF7 IgM concentration in serum from tumor-bearing rats, we found that immune serum was more effective at lysing unsensitized MCF7 cells; serum from naïve and tumor-bearing rats at a concentration of 40% resulted in 12% +/-2.5% and 18% +/- 3.2% lysis, respectively (mean +/- SD, n=4, $P<0.05$).

Discussion

Endogenously expressed DAF and CD59 provide the human breast cancer cell line MCF7 with resistance to human complement deposition and lysis in vitro, even in the presence of complement activating anti-MCF7 antibodies. However, MCF7 cells are susceptible to rat complement in vitro, illustrating the fact that human complement inhibitors are not effective against rat complement. The resistance of the rat CD59 and Crry transfected MCF7 cells to rat complement parallels the resistance of MCF7 cells to human complement and establishes the clinical relevance of using these transfected cells to study complement inhibitors and complement effector mechanisms in rats. For studies on complement, we consider the rat a better model than the mouse, since isolated mouse serum contains very low levels of hemolytic complement. The reason for this may be strain dependent and has been variously ascribed to deficiencies in different terminal complement proteins and the inability of the mouse classical pathway C5 convertase to cleave C5 and initiate the terminal pathway (22-25).

MCF7 tumorigenicity in nude rats was dramatically enhanced when C3 deposition is inhibited, but inhibition of cytolytic MAC formation alone had no effect on tumor growth. We infer from these results that only cell-mediated mechanisms promoted or enhanced by the deposition of C3 fragments generated during the complement activation phase are involved in controlling the growth of MCF7. Complement activation on a tumor cell will result in the deposition of activation products C3b and its longer lived degradation product iC3b. Only C3b can participate further in amplification of the complement cascade, but both proteins are ligands for complement receptors found on immune effector cells in rats and humans. Crry (DAF and MCP in humans), but not CD59 will control C3 opsonization. Complement receptor 1 (CR1) is expressed on phagocytes and binds C3b with high affinity and iC3b (and C4b, a classical pathway fragment) with low affinity. Complement receptor 3 (CR3 or CD11b/CD18) binds iC3b with high affinity and is expressed on phagocytes and NK cells. These C3 receptors, in particular CR3, can promote the adhesion of C3-opsonized tumor cells with effector cells, can promote and enhance ADCC/NK lysis and can play an important role in leukocyte adhesion and migration during an inflammatory process (for review of C3 receptors see ref. (26)). The complement activation product C5a, the generation of which will not be affected by CD59, may also contribute to cell-mediated effector functions. C5a is a chemoattractant and a powerful mediator of inflammation that may potentiate an anti-tumor response. It should nevertheless be noted that compared to human cell types, complement-mediated recruitment of rat leukocytes and associated rat complement receptors are less well defined and represent a topic for further study. As evidenced by the lack of any effect of rat CD59 expression on MCF7 tumorigenesis, the terminal cytolytic phase of the complement pathway does not alone play any direct role in controlling tumor growth in this model, although there was some synergy when both Crry and CD59 were expressed together on MCF7 cells. Interestingly, a previous study indicated an important role for CD59 in the survival of a neuroblastoma cell line in rats (21), indicating that different complement-associated mechanisms operate in controlling growth of different tumors. The reason for this difference has not been investigated, but could be due to several factors including relative levels of endogenous complement inhibitors, differences in quantity or isotype of tumor-reactive antibodies present in nude rat serum and differential susceptibility to ADCC and NK cell lysis.

Complement was deposited on MCF7 cells grown in rats although complement activating anti-MCF7 antibodies were not exogenously administered. This observation is explained by the finding that nude rat serum contained naturally occurring IgG and IgM reactive to MCF7 cells. There was also an anti-MCF7 IgM immune response in tumor-bearing rats. IgM is an effective activator of complement, but only IgG will bind to Fc receptors on phagocytes and NK cells. MCF7 cells express the tumor-associated antigen $MUC1^+$ and, interestingly, antibodies to MUC1 occur naturally in healthy individuals (27-29). Further, as in our rat model, an IgM humoral immune response occurs in patients with $MUC1^+$ tumors (28-30) and deposited complement has also been detected in tissue samples from breast and other tumors (2, 4). Thus, the data reported here raise the possibility that regulating the function of complement inhibitors of activation on a tumor cell surface may augment the effector phase of a normally ineffective humoral immune response to MUC1 antigen, or indeed to other tumor antigens. Enhancing C3 deposition by suppressing complement inhibitor function may also enhance the induction phase of a humoral immune response, since C3 opsonization of antigen can provide a costimulatory signal via complement receptor 2 (CR2/CD21) expressed on B cells (31). In this context, enhancing C3 deposition by suppressing complement inhibitor function may also be a useful strategy to potentiate a tumor vaccine strategy. CR2 is also expressed on some T cells, although a role for CR2 in T-cell immunity has not been reported.

Whether or not tumor expressed complement inhibitors and their blockade can effect the outcome of an immune response, the data provide strong direct in vivo evidence to support a hypothesis that inhibiting DAF and/or MCP will enhance the outcome of immunotherapy of certain tumors using complement activating anti-tumor antibodies. The species selective activity of complement inhibitors and the data presented here also provide an explanation for the disappointing results obtained in the clinic with antibodies that were successful at treating tumors in rodent models of human cancer.

At the present time, blocking the function of complement inhibitors specifically on tumor cells in vivo is a technically difficult proposition, although significant progress is being made in cell targeting strategies. Nevertheless, the specificity required for targeting is less critical than it would be for targeting toxins for example, since blocking the function of complement inhibitors on normal tissue is unlikely to present a significant health risk in the absence of a specific complement activating signal (such as anti-tumor antibody). Of potential significance, a monoclonal antibody that has been used successfully for tumor imaging was recently found to be specific for DAF (32). The expression of DAF on normal tissue raises the question of how it was possible to successfully image with this antibody and why only background levels of the antibody were found on endothelium and blood cells. A possible explanation for this finding comes from a more recent study showing that DAF is upregulated by as much as 100-fold on some tumors (11). These findings may bode well for strategies aimed at tumor-targeted regulation of this complement inhibitor of activation.

Figure legends

Figure 1. Analysis of rat complement inhibitor expression on transfected MCF7 cells. Stably transfected MCF7 cells were sorted and isolated populations analyzed by flow cytometry as shown in figure. Rat CD59 and Crry expression was detected using FITC and PE conjugated antibodies, respectively. Relative fluorescence shown on each axis.

Figure 2. Susceptibility of transfected MCF7 cells to lysis by rat complement. Antibody sensitized MCF7 cells transfected with rat CD59, rat Crry, both complement inhibitors, or mock transfected MCF7 were incubated in the indicated concentration of rat serum and lysis determined (mean +/- SD, n=5).

Figure 3. Deposition of rat C3 on transfected MCF7 cells. MCF7 cells transfected with rat CD59, rat Crry, or mock transfected MCF7 were sensitized with increasing concentrations of anti-MCF7 antiserum and incubated with 10% C6-deficient rat serum. Rat C3 deposition was measured by flow cytometry. Figure is representative of 5 separate determinations.

Figure 4. Tumor incidence in nude rats inoculated with MCF7 cells transfected with different rat complement inhibitors. Rats were inoculated with 5×10^6 cells and occurrence of tumors recorded at day 60 post inoculation. Combined data from 3 separate experiments is shown.

Figure 5. Growth curves of MCF7 cells transfected with different rat complement inhibitors in nude rats. Rats were inoculated with 5×10^6 mock transfected MCF7 cells or MCF7 cells transfected with rat CD59, rat Crry, or both rat CD59 and Crry. Growth was measured at intervals for 60 days. Combined data from 3 separate experiments is shown (mean +/- SD, n= 14-20 {refer to fig. 4}).

Figure 6. Analysis of tumor-derived MCF7 cells by flow cytometry. Tumor cells obtained from rats inoculated with either rat CD59 or Crry transfected MCF7 cells were analyzed for continued expression of rat complement inhibitor. Tumor-derived mock transfected MCF7 were also analyzed for the continued endogenous expression of MUC1 antigen and for deposition of rat IgG and IgM. Analyses were performed on cells derived from tumors 28-30 days after inoculation. Panels show data obtained with isotype control antibody (gray trace) and specific antibody (black trace).

Figure 7. Relationship between Crry expression and C3 deposition on tumor-derived transfected and mock transfected MCF7 cells. Cell suspensions obtained from MCF7 tumors were analyzed by two color flow cytometry using FITC and PE conjugated anti-C3 and anti-Crry antibody, respectively. Relative fluorescence shown on each axis.

Figure 8. Deposition of rat IgG and IgM on tissue culture-derived MCF7 cells. MCF7 were incubated in heat inactivated serum obtained from naïve (gray trace) or tumor-bearing rats (black trace) and analyzed separately for either IgG or IgM deposition by flow cytometry. Control antibodies gave a mean fluorescence < 10.

References

1. Clynes, R. A., Towers, T. L., Presta, L. G., and Ravetch, J. V. Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets, Nat Med. *6:* 443-6, 2000.
2. Niculescu, F., Rus, H. G., Retegan, M., and Vlaicu, R. Persistent complement activation on tumor cells in breast cancer, Am J Pathol. *140:* 1039-43, 1992.
3. Magyarlaki, T., Mosolits, S., Baranyay, F., and Buzogany, I. Immunohistochemistry of complement response on human renal cell carcinoma biopsies, Tumori. *82:* 473-9., 1996.
4. Lucas, S. D., Karlsson-Parra, A., Nilsson, B., Grimelius, L., Akerstrom, G., Rastad, J., and Juhlin, C. Tumor-specific deposition of immunoglobulin G and complement in papillary thyroid carcinoma, Hum Pathol. *27:* 1329-35., 1996.
5. Kim, Y. U., Kinoshita, T., Molina, H., Hourcade, D., Seya, T., Wagner, L. M., and Holers, V. M. Mouse complement regulatory protein Crry/p65 uses the specific mechanisms of both human decay-accelerating factor and membrane cofactor protein, Journal of Experimental Medicine. *181:* 151-159, 1995.
6. Takizawa, H., Okada, N., and Okada, H. Complement inhibitor of rat cell membrane resembling mouse Crry/p65, J.Immunol. *152:* 3032-3038, 1994.
7. Quigg, R. J., Lo, C. F., Alexander, J. J., Sneed, A. E. d., and Moxley, G. Molecular characterization of rat Crry: widespread distribution of two alternative forms of Crry mRNA, Immunogenetics. *42:* 362-367, 1995.
8. Rollins, S. A. and Sims, P. J. The complement-inhibitory activity of CD59 resides in its capacity to block incorporation of C9 into membrane C5b-9, J.Immunol. *144:* 3478-3483, 1990.
9. Meri, S., Morgan, B. P., Davies, A., Daniels, R. H., Olavesen, M. G., Waldemann, H., and Lachmann, P. J. Human protectin (CD59), an 18-20 kD complement lysis restricting factor, inhibits C5b-8 catalysed insertion of C9 into lipid bilayers, Immunol. *72:* 1-9, 1990.

10. Kinders, R. J., Jones, T., Root, R., Bruce, C., Murchison, H., Corey, M., Williams, L., Enfield, D., and Hass, G. M. Complement factor H or a related protein is a marker for transitional cell cancer of the bladder, Clin.Cancer Res. *4:* 2511-2520, 1998.

11. Li, L., Spendlove, I., Morgan, J., and Durrant, L. G. CD55 is over-expressed in the tumour environment, Br J Cancer. *84:* 80-86., 2001.

12. Maio, M., Brasoveanu, L. I., Coral, S., Sigalotti, L., Lamaj, E., Gasparollo, A., Visintin, A., Altomonte, M., and Fonsatti, E. Structure, distribution, and functional role of protectin (CD59) in complement-susceptibility and immunotherapy of human malignancies (Review), Int.J.Oncol. *13:* 305-318, 1998.

13. Gorter, A. and Meri, S. Immune evasion of tumor cells using membrane-bound complement regulatory proteins, Immunology Today. *20:* 576-582, 1999.

14. Jurianz, K., Ziegler, S., Garcia-Schuler, H., Kraus, S., Bohana-Kashtan, O., Fishelson, Z., and Kirschfink, M. Complement resistance of tumor cells: basal and induced mechanisms, Mol Immunol. *36:* 929-39., 1999.

15. Rushmere, N. K., Harrison, R. A., van der Berg, C. W., and Morgan, B. P. Molecular cloning of the rat analogue of human CD59: structural comparison with human CD59 and identification of a putative active site, Biochem.J. *304:* 595-601, 1994.

16. Yu, J., Abagyan, R. A., Dong, S., Gilbert, A., Nussenzweig, V., and Tomlinson, S. Mapping the active site of CD59, Journal of Experimental Medicine. *185:* 745-753, 1997.

17. Harlow, E. and Lane, D. Antibodies. A laboratory manual. New York: Cold Spring Harbor Laboratory, 1988.

18. Sakurada, C., Seno, H., Dohi, N., Takizawa, H., Nonaka, M., Okada, N., and Okada, H. Molecular cloning of the rat complement regulatory protein, 5I2 antigen, Biochem Biophys Res Commun. *198:* 819-26., 1994.

19. Yu, J., Caragine, T., Chen, S., Morgan, B. P., Frey, A. F., and Tomlinson, S. Protection of human breast cancer cells from complement-mediated lysis by expression of heterologous CD59., Clin.Exp.Immunol. *115:* 13-18, 1999.

20. Hakulinen, J. and Meri, S. Expression and function of the complement membrane attack complex inhibitor protectin (CD59) on human breast cancer cells, Laboratory Investigation. *71:* 820-827, 1994.

21. Chen, S., Caragine, T., Cheung, N. K., and Tomlinson, S. CD59 Expressed on a Tumor Cell Surface Modulates DAF Expression and Enhances Tumor Growth in a Rat Model of Human Neuroblastoma, Cancer Res. *60:* 3013-3118, 2000.

22. Ebanks, R. O. and Isenman, D. E. Mouse complement component C4 is devoid of classical pathway convertase subunit activity, J.Immunol. *33:* 297-309, 1996.

23. Ooi, Y. M. and Colten, H. R. Genetic defect in secretion of complement C5 in mice, Nature. *282:* 207-8., 1979.

24. Ong, G. L. and Mattes, M. J. Mouse strains with typical mammalian levels of complement activity, J.Immunol.Meth. *125:* 147-158, 1989.

25. Tanaka, S., Suzuki, T., and Nishioka, K. Assay of classical and alternative pathway activities of murine complement using antibody-sensitized rabbit erythrocytes, J.Immunol.Meth. *86:* 161-170, 1986.

26. Delibrias, C., Fischer, E., and Kazachtkine, M. D. Receptors for human C3 fragments. *In:* K. Rother, G. O. Till, and G. M. Haensch (eds.), the Complement System, 2nd edition, pp. 211-220. Berlin: Springer-Verlag, 1997.

27. von Mensdorff-Pouilly, S., Petrakou, E., Kenemans, P., van Uffelen, K., Verstraeten, A. A., Snijdewint, F. G., van Kamp, G. J., Schol, D. J., Reis, C. A., Price, M. R., Livingston, P. O., and Hilgers, J. Reactivity of natural and induced human antibodies to MUC1 mucin with MUC1 peptides and n-acetylgalactosamine (GalNAc) peptides, Int J Cancer. *86:* 702-12., 2000.

28. Richards, E. R., Devine, P. L., Quin, R. J., Fontenot, J. D., Ward, B. G., and McGuckin, M. A. Antibodies reactive with the protein core of MUC1 mucin are present in ovarian cancer patients and healthy women, Cancer Immunol Immunother. *46:* 245-52., 1998.

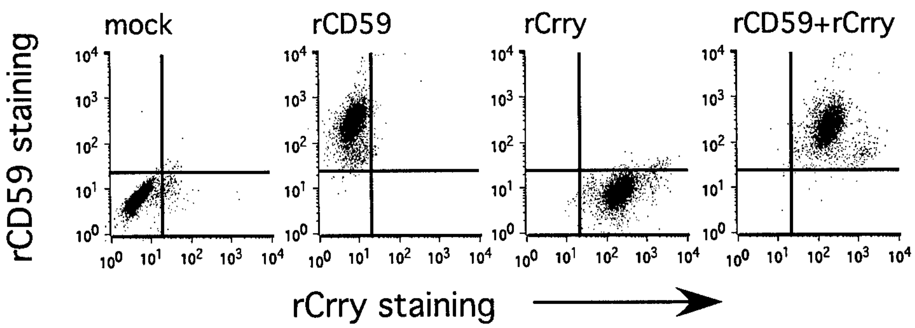
29. von Mensdorff-Pouilly, S., Verstraeten, A. A., Kenemans, P., Snijdewint, F. G., Kok, A., Van Kamp, G. J., Paul, M. A., Van Diest, P. J., Meijer, S., and Hilgers, J. Survival in early breast cancer patients is favorably influenced by a natural humoral immune response to polymorphic epithelial mucin, J Clin Oncol. *18:* 574-83., 2000.

30. Kotera, Y., Fontenot, J., Pecher, G., Metzger, R., and Finn, O. Humoral immunity against a tandem repeat epitope of human mucin MUC-1 in sera from breast, pancreatic, and colon cancer patients, Cancer Res. *54:* 2856-2860, 1994.

31. Carroll, M. C. and Fischer, M. B. Complement and the immune response, Curr Opin Immunol. *9:* 64-9., 1997.

32. Spendlove, I., Li, L., Carmichael, J., and Durrant, L. G. Decay accelerating factor (CD55): A target for cancer vaccines, Cancer Res. *59:* 2282-2286, 1999.

fig. 1.



mock
rCD59
rCrry
rCD59+rCrry
rCD59 staining
rCrry staining

Fig. 2

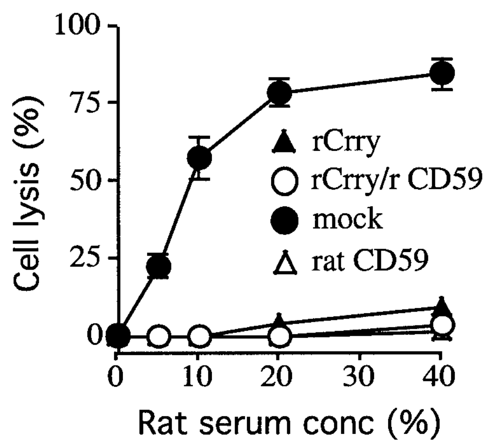
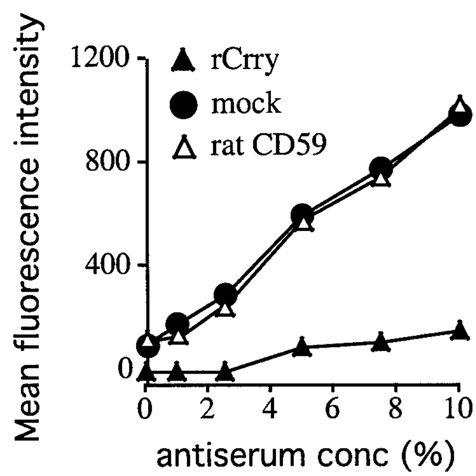
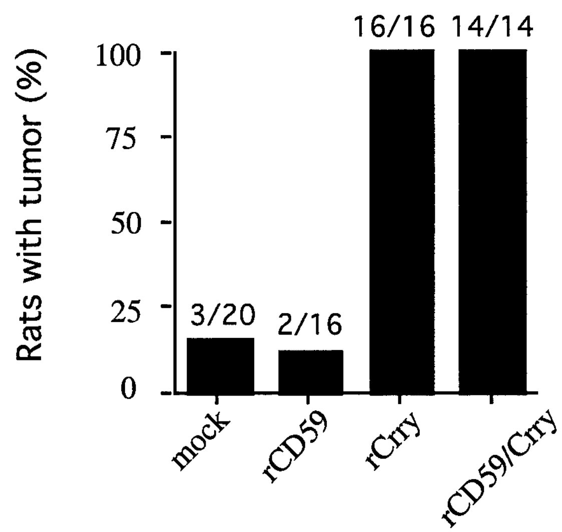

100
75
50
25
0
Cell lysis (%)
rCrry
rCrry/r CD59
mock
rat CD59
0
10
20
30
40
Rat serum conc (%)

Fig. 3



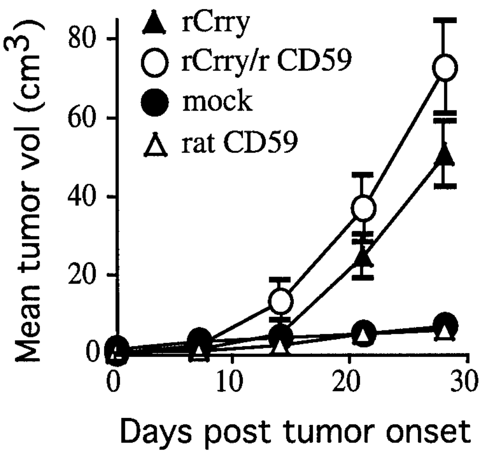
1200
800
400
0
0
2
4
6
8
10
rCrry
mock
rat CD59
Mean fluorescence intensity
antiserum conc (%)

Fig. 4



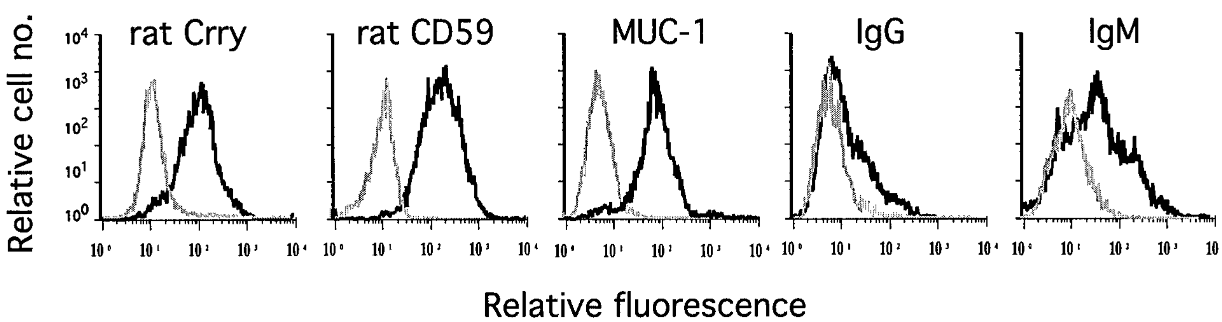
Rats with tumor (%)
100
75
50
25
0
3/20
2/16
16/16
14/14
mock
rCD59
rCrry
rCD59/Crry

Fig. 5



rCrry
rCrry/r CD59
mock
rat CD59
Mean tumor vol (cm³)
80
60
40
20
0
0
10
20
30
Days post tumor onset

Fig. 6



rat Crry
rat CD59
MUC-1
IgG
IgM
Relative cell no.
Relative fluorescence

Fig. 7

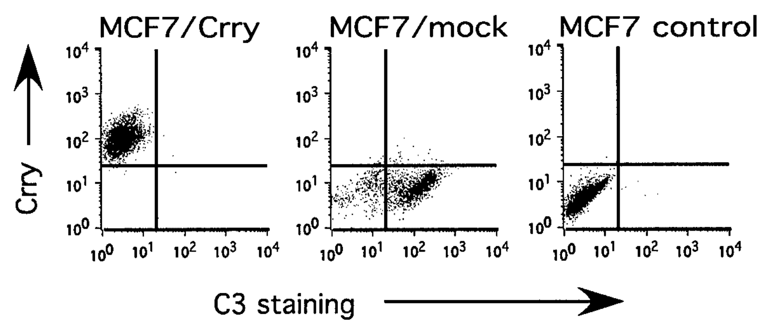
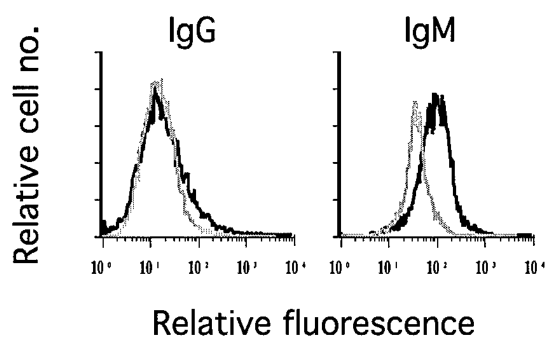


Fig. 8



IgG
IgM
Relative cell no.
10^0
10^1
10^2
10^3
10^4
10^0
10^1
10^2
10^3
10^4
Relative fluorescence